

The mouse tyrosinase promoter is sufficient for expression in melanocytes and in the pigmented epithelium of the retina

(chloramphenicol acetyltransferase expression/cell lineage/melanoma cells/neural crest/transgenic mice)

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ABSTRACT The mouse *c* locus encodes tyrosinase (monophenol monooxygenase; monophenol, L-dopa:oxygen oxidoreductase, EC 1.14.18.1), the key enzyme in melanin synthesis, which is expressed in the pigment epithelium of the retina and in melanocytes derived from the neural crest. To define regulatory regions of the gene that are important for cell type-specific expression, a deletion series of the tyrosinase 5' region was fused to a chloramphenicol acetyltransferase (CAT) reporter gene and electroporated into tyrosinase-expressing and -nonexpressing cell lines. We show that 270 base pairs 5' of the transcriptional start site is sufficient for CAT expression in a human and a mouse melanoma cell line. This 5' flanking fragment, when cloned in the context of a tyrosinase minigene construct and injected into fertilized eggs of an albino mouse strain, is sufficient for cell type-specific expression in mice. The transgenic mice were pigmented in both skin and eyes. *In situ* hybridization analysis shows that the 270-base-pair regulatory region contains elements sufficient for specific expression of the transgene both in the pigmented epithelial cells of the retina, which are derived from the optic cup, and in neural crest-derived melanocytes.

Tyrosinase (monophenol monooxygenase; monophenol, L-dopa:oxygen oxidoreductase, EC 1.14.18.1) is the essential enzyme in melanogenesis (1, 2). The tyrosinase gene is expressed in pigmented cells of different origin. Whereas melanocytes residing in skin, iris, and choroid are neural crest derivatives, pigmented cells of the outmost layer of the retina, the pigmented epithelium, are derived from the optic cup (3, 4). In the mouse, the structural gene for tyrosinase is encoded by the genetically well-characterized *c* locus (1, 24, 25). This has been demonstrated more recently by rescue of the *c* locus mutant albino (*c/c*) after introduction of a functional tyrosinase gene into mice (5, 6, 26); 5.5 kilobases (kb) of 5' flanking mouse tyrosinase sequence directed expression of the transgene in a cell type-specific manner (5).

We are interested in defining the region of the 5' sequence important for cell type-specific expression. Thus, a deletion series of the 5' region of the tyrosinase gene was fused to a chloramphenicol acetyltransferase (CAT) reporter gene (TyrCAT constructs) and transfected into tyrosinase-expressing and -nonexpressing cell lines. Melanoma cell-specific expression was obtained with as little as 270 base pairs (bp) of 5' flanking sequence, indicating the presence of cis-regulatory elements determining expression of the tyrosinase gene in melanoma cells. To establish whether these sequences are also sufficient to direct cell type-specific and developmentally correct expression of the tyrosinase gene in animals (i.e., the pigmented epithelium of the retina and the melanocytes derived from the neural crest), we microinjected a

tyrosinase minigene containing only 270 bp of 5' flanking sequence into fertilized eggs of an albino mouse strain. The resulting transgenic mice had pigmented skin and eyes. *In situ* hybridization to sections of skin and eyes showed cell type-specific expression of the transgene both in neural crest-derived melanocytes and in cells of the pigmented epithelium. We therefore conclude that the cis elements mediating cell type-specific expression in these two lineages are located within 270 bp upstream of the transcription start site.

MATERIALS AND METHODS

Construction of Plasmids. All TyrCAT constructs are based on the CAT expression vector pBLCAT6 (M. Boshart, M.K., A. Schmidt, G.S., and B. Luckow; unpublished data), which is derived from pBLCAT3 (7). The TyrCAT 5' series was constructed by inserting various restriction fragments derived from pXho23 into the polylinker of pBLCAT6. pXho23 contains tyrosinase genomic sequences from -7.8 kb to +145 bp, inserted into pBluescript M13 (Stratagene). For convenient cloning, a *Sma* I restriction site was introduced at position +9 of the tyrosinase sequence (8) by site-directed mutagenesis (9) and was used for cutting out the various restriction fragments [pTyrCAT (-6.1 kb), *Asp*718/*Sma* I; pTyrCAT (-3.7 kb), *Pst* I/*Sma* I; pTyrCAT (-0.27 kb), *Xba* I/*Sma* I; pTyrCAT (-0.08 kb), *Hind*III/*Sma* I]. Further details on the cloning of the constructs are available on request.

To construct the tyrosinase minigene ptrTyr5, an 11-kb *Xba* I/*Mlu* I fragment derived from ptrTyr4 (5) was cloned into a modified pBluescript M13 vector (Stratagene) containing a *Bss*HII cloning site (S.R., unpublished data). The minigene was recovered for microinjection as an 11-kb *Not* I/*Sal* I fragment.

Cell Culture and Electroporation of Cells. The human melanoma cell lines SKMel25 and SKMel28 (10) were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium. NIH 3T3 cells and the mouse melanoma cell line B16 were cultured in DMEM. Media were supplemented with 10% fetal calf serum. Electroporation was performed with a Gene Pulser (Bio-Rad) and capacitance extender (Bio-Rad) essentially as described (11) at a concentration of 2.5×10^7 cells per ml (SKMel25, SKMel28, B16) or 1.25×10^7 cells per ml (NIH 3T3). A luciferase expression vector (RSVlucA/H) was cotransfected (12) and used as internal reference. The CAT and luciferase assays were performed as described elsewhere (10), except that the cell extracts were prepared in 140 μ l of 250 mM Tris-HCl, pH 7.8/1 mM EDTA. Each transfection was performed at least in duplicate.

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Abbreviation: CAT, chloramphenicol acetyltransferase.

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		human		mouse	
		SKMel28	SKMel25	B16	NIH3T3
pBLCAT6	CAT	1	1	1	1
pTyrCAT (-0.08)	-80 bp CAT	10	9	10	4
pTyrCAT (-0.27)	-270 bp CAT	164	20	95	7
pTyrCAT (-3.7)	-3.7 kb CAT	294	11	213	8
pTyrCAT (-6.1)	-6.1 kb CAT	432	16	351	1

FIG. 1. Determination of melanoma cell-specific activity of the tyrosinase gene promoter by 270 bp of 5' flanking sequence. The tyrosinase CAT fusion genes were transfected into the different cell lines by electroporation. Cellular extracts were prepared after 24 hr and assayed for CAT and luciferase activity. The CAT activity level of pBLCAT6 was 2.04 pmol·min⁻¹·mg⁻¹, 0.77 pmol·min⁻¹·mg⁻¹, 1.59 pmol·min⁻¹·mg⁻¹, and 0.76 pmol·min⁻¹·mg⁻¹ for SKMel28, SKMel25, B16, and NIH 3T3, respectively. CAT activity (luciferase corrected) was calculated as relative activity by arbitrarily setting the activity of pBLCAT6 to 1.

Transgenic Mice. After removal of vector sequences by cleavage with *Not* I and *Sal* I, the minigene was isolated from an agarose gel and purified with glass powder (Geneclean; Bio 101) followed by dialysis against injection buffer (10 mM Tris·HCl, pH 7.5/0.1 mM EDTA) on dialysis filters (Millipore). Procedures for microinjection and manipulation of fertilized eggs obtained from NMRI mice were as described (5). Transgenic founder mice were identified by Southern blot analysis of DNA extracted from tail biopsies of 3-week-old mice.

In Situ Hybridization. Skin and eyes of both nontransgenic and transgenic littermates (4 days old) were isolated, washed in phosphate-buffered saline, and immediately fixed in 4% paraformaldehyde. *In situ* hybridization experiments were carried out on paraffin-embedded tissue sections essentially as described (13, 14) using ³⁵S-labeled antisense and sense RNA probes generated from pmcTyr54 or pSV-H (see Fig. 2) (5). After 1–2 weeks of exposure, the sections were developed with Kodak D19 and stained with hematoxylin and eosin.

RESULTS

A 270-bp Fragment Is Sufficient to Direct Specific Expression in Melanoma Cells. To identify cis-acting elements important for cell type-specific expression of the tyrosinase gene, we performed transfer of TyrCAT fusion genes into a human melanoma (SKMel28) and a mouse melanoma cell line (B16) that express tyrosinase. Two cell lines that are tyrosinase negative (human melanoma SKMel25; mouse fibroblasts NIH 3T3) and in which no detectable level of tyrosinase mRNA is found were used as controls (8, 10, 15–17).

Since we previously had shown that 5.5 kb of flanking sequence of the tyrosinase gene is sufficient for expression in transgenic mice (5), we introduced fusion genes with 5' flanking sequence of different length up to 6.1 kb. The fusion gene containing 6.1 kb of 5' flanking sequence was specifically expressed in both the human and the mouse tyrosinase-positive cell lines (Fig. 1). Transfection of smaller constructs revealed that the most dramatic decrease in expression of CAT activity occurs between the -270- and -80-bp constructs (SKMel28, 16-fold; B16, 9.5-fold). The 270 bp of 5' flanking sequence gave much higher levels of CAT expression in the two tyrosinase-positive cell lines (SKMel28 and B16) than in the two tyrosinase-negative cell lines (SKMel25 and NIH 3T3). Comparing the CAT activities of the tyrosinase fusion genes with a CAT expression plasmid containing the thymidine kinase promoter (pBLCAT2; see ref. 7), we conclude that the mouse tyrosinase gene has a relatively weak but cell type-specific promoter (data not shown).

Rescue of the Albino Phenotype by Generating Transgenic Mice Carrying a Tyrosinase Minigene. Are the element(s)

required for melanoma-specific expression sufficient to direct cell type-specific expression in mice? We are particularly interested in this question since the same gene is responsible for pigmentation in cells of different origin—i.e., melanocytes derived from the neural crest and cells of the pigment epithelium of the retina, which are derived from the optic cup. We therefore designed the construct ptrTyr5 (Fig. 2), which contains the first 270 bp (*Xba* I site) of the tyrosinase 5' region, the full coding region of the tyrosinase gene interrupted by the first intron, and a simian virus 40 splice and polyadenylation site. The minigene was separated from vector sequences and injected into fertilized eggs from the albino mouse strain NMRI (*c/c*). Then 184 injected embryos were transferred to both oviducts of 6 pseudopregnant females, of which 4 became pregnant and gave rise to 17 offspring. By DNA analysis, 5 transgenic animals were identified (data not shown). Four mice had pigmented skin and eyes and 2 of those transmitted the transgene to offspring (line 331, 7 transgenic offspring of 13 born; line 334, 3 of 10), which were also pigmented. One transgenic animal showed no obvious signs of pigmentation and was not analyzed further. We conclude that we have obtained pigmentation in mice by introduction of a functional tyrosinase gene, the expression of which is mediated by only 270 bp of upstream sequence.

Cell Type-Specific Expression of the Tyrosinase Minigene in Transgenic Mice. To verify that pigmentation in skin and eye is due to cell type-specific expression of the transgene, we performed *in situ* hybridization analyses on tissue sections from skin and eyes of 4-day-old nontransgenic and transgenic mice. As shown in Figs. 3–5, expression of the transgene displays the same cell type-specific pattern as the endogenous tyrosinase gene.

Fig. 3 shows specific hybridization of the tyrosinase probe detecting the endogenous gene and the transgene in the iris of

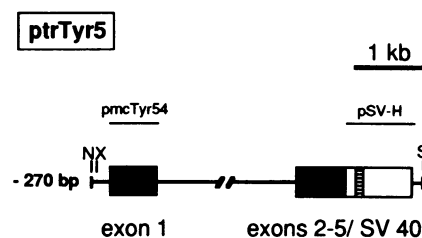


FIG. 2. The tyrosinase minigene used for injection. The components of ptrTyr5 are as follows: thin line, 5' upstream region and first intron of the tyrosinase gene; solid box, exons 1–5 of the mouse tyrosinase gene; open box, simian virus 40 (SV40) sequences containing splice and polyadenylation sites; hatched box, 66-bp small T intron of SV40. The probes pmcTyr54 and pSV-H used for *in situ* hybridization are indicated. N, *Not* I; S, *Sal* I; X, *Xba* I.

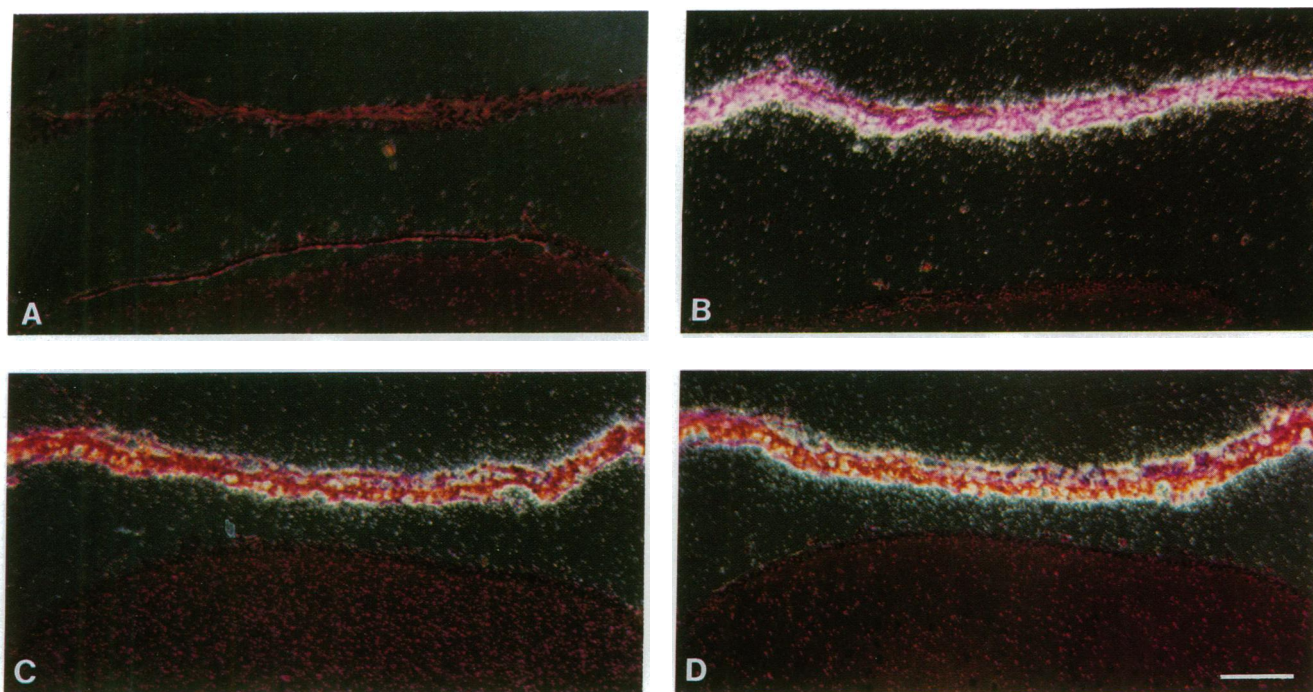


FIG. 3. Localization of tyrosinase mRNA and transgene mRNA in the iris. Sections from a nontransgenic NMRI (albino) mouse (A and B) and from a transgenic mouse (line 331; C and D) were hybridized to 35 S-labeled RNA probes: the transgene-specific probe pSV-H (A and C) and the tyrosinase probe pmcTyr54 (B and D; see Fig. 2). No hybridization was obtained when the transgene-specific probe was used on sections of eyes from nontransgenic mice (A). Note that the tyrosinase gene-specific probe detects both endogenous and transgene-specific transcripts (B and D). Dark-field photomicrographs. (Bar = 50 μ m.)

both the nontransgenic (Fig. 3B) and the transgenic (Fig. 3D) mouse. When the transgene-specific probe pSV-H was used, the iris of the transgenic mouse revealed the same grain distribution, thus indicating specific expression of the trans-

gene (Fig. 3C). As expected, no signals were observed with this probe in the nontransgenic mouse (Fig. 3A).

In the back of the eye, expression from the transgene has resulted in the production of pigment in cells of the choroid

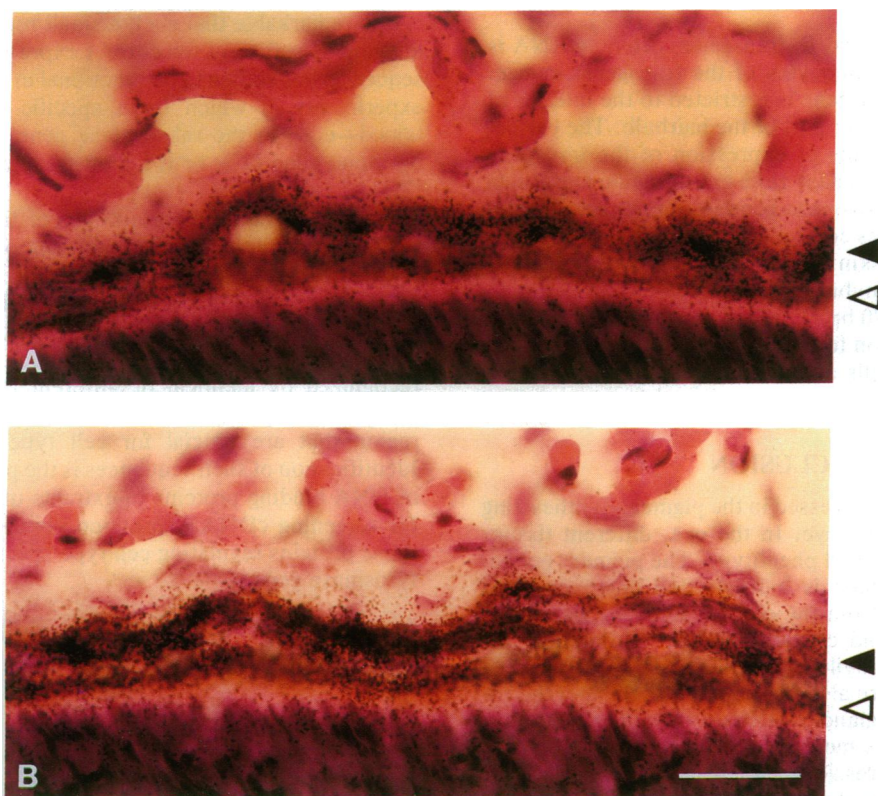


FIG. 4. The transgene is expressed in choroid and pigmented retina. Sections from the back of the eye (transgenic mouse) were hybridized to the transgene-specific probe pSV-H (A) or the tyrosinase-specific probe pmcTyr54 (B). Hybridization was restricted to pigmented cells of the choroid (arrowhead) and the pigment epithelium of the retina (open arrowhead). Bright-field photomicrographs. (Bar = 20 μ m.)

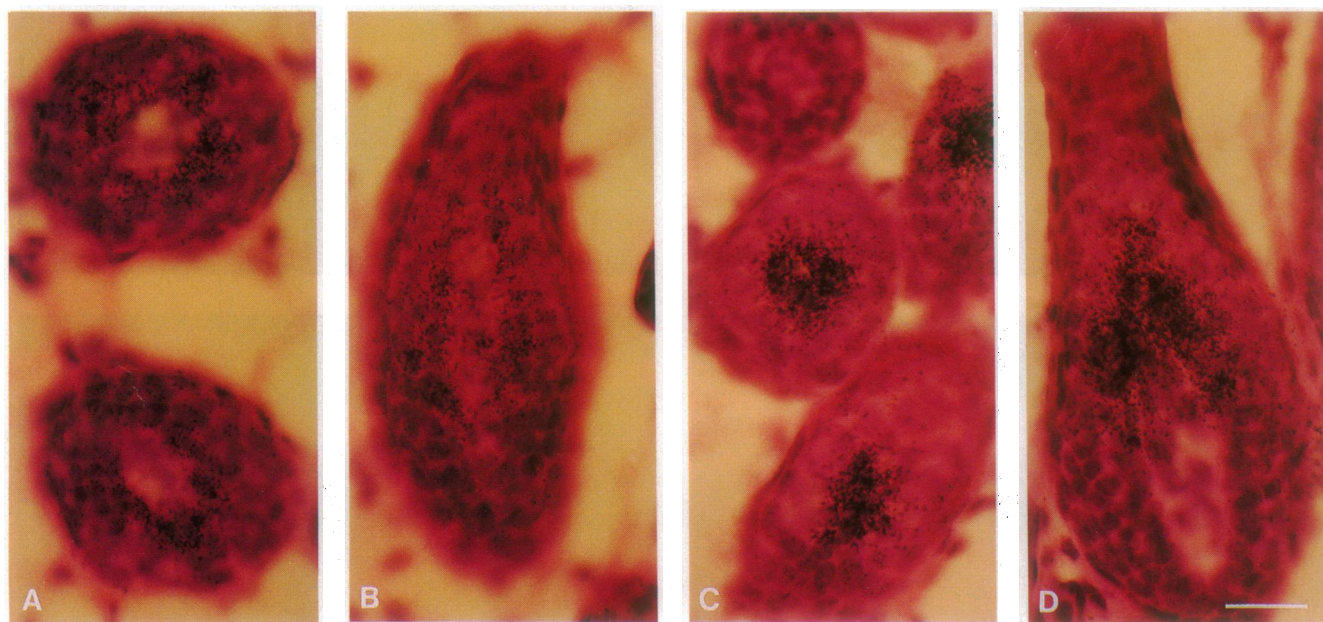


FIG. 5. The transgene is specifically expressed in melanocytes of the skin. (A and B) Transgene-specific transcripts in the hairbulbs of a transgenic mouse. (C and D) Tyrosinase-specific transcripts in the hairbulbs of a nontransgenic albino NMRI mouse. Bright-field photomicrographs. (Bar = 15 μ m.)

(neural crest derived) and the pigmented epithelium of the retina (derived from the optic cup; Fig. 4). Hybridization with the transgene-specific probe (Fig. 4A) and the tyrosinase probe (Fig. 4B) reveals specific expression of the transgene in these two layers. As revealed by this *in situ* hybridization analysis, the sites of expression of the endogenous gene and the transgene are identical in a transgenic and a nontransgenic mouse (5).

As shown in Fig. 5 A and B, transgene-specific mRNA is also present in skin melanocytes of the transgenic mouse. Transcription of the transgene is restricted to the interior of the root sheath at the lower part of the hairbulb. The perfect concordance between sites of expression of the transgene (Fig. 5 A and B), the tyrosinase gene in transgenic mice (data not shown), and the tyrosinase gene in nontransgenic mice (Fig. 5 C and D) indicates correct expression of the transgene in melanocytes of the skin.

In summary, *in situ* hybridization indicates that the mini-gene containing only 270 bp of 5' flanking sequence provides all important information for cell type-specific expression in pigmented epithelial cells and neural crest-derived melanocytes.

CONCLUSION

The tyrosinase gene is expressed in the pigment-synthesizing cells of the skin and the eye. In the eye, different tissues contribute to the pigmented phenotype. The pigment epithelium of the retina, which is a single cell layer, contains pigment cells derived from the outer wall of the optic cup. The iris and the choroid contain neural crest-derived melanocytes, which are heavily pigmented (3, 4). Neural crest-derived melanocytes are also found in the cochlea of the ear (18) and the Harderian gland, which is situated behind the eye and contains numerous melanocytes lying around the acini (19). In the skin, expression of tyrosinase is restricted to neural crest-derived melanocytes residing in the lower part of the hairbulb. This differential expression is characterized by a specific temporal pattern—i.e., pigmentation in the pigment epithelium is detectable much earlier (at around day 11–12

p.c.) than in melanocytes of skin or choroid (around birth; ref. 20).

Here we have addressed the question of which sequences are required for cell type-specific expression of the tyrosinase gene. The results show that 270 bp of upstream sequence is sufficient for specific expression in melanoma cell lines and in the whole animal in both types of pigmented cells.

It is remarkable that such a small region directly flanking the gene is sufficient for correct expression. A similar short sequence was shown to be functional in only a few transgenic experiments in which tissue-specific expression was obtained—e.g., in the rat elastase gene (21), the rat growth hormone gene (22), or the mouse pancreatic amylase gene (23).

The allocation of important information to such a defined region will facilitate precise mapping of single cis elements responsible for cell type-specific expression. It remains an open question whether cell type-specific expression in cells of the pigmented epithelium and melanocytes of the choroid is mediated by different cis elements or the same cis element recognized by identical or different factors. We can now define the sequences within the 270-bp (from –270 to –80 bp) region that are crucial for cell type-specific expression. Identification of such sequences is the prerequisite for search of DNA binding proteins in extracts of melanoma cells and for screening cDNA expression libraries to finally identify those proteins regulating cell type-specific expression of the tyrosinase gene.

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